Confocal Application Notes
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FRET Sensitized Emission (SE) in LASAF

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1- Overview on FRET:

Fluorescence Resonance Energy Transfer or Förster Resonance Energy Transfer (FRET) is a technique allowing study of proteins interactions, which are in proximities but beyond light microscopy resolution (typical distances of 1-10 nm). Originally measured by fluorescence spectroscopy, FRET can also be measured by fluorescence microscopy. Since FRET occurs over distances similar to the size of proteins, it can be used to extend the resolution of the fluorescence microscope (typically >250 nm) to detect protein-protein interactions. Hence, it is an ideal approach to determine whether proteins that are co-localized at the level of light microscopy really interact with one another.

Method: FRET involves a non-radiative transfer of energy from an excited state donor fluorophore to a nearby acceptor. The energy transfer efficiency $FRET_{eff}$ is directly related to the distance r separating a given donor and acceptor pair by

$$FRET_{eff}=1/[1+(r/R_0)^6]$$

The resolution of FRET is thus defined by R_0 which is typically < 10-70 Å. R_0 depends of the extent of overlap between the donor emission and the acceptor spectra, the absorption coefficient for the acceptor, the quantum yield of the donor, and the relative orientation of the donor and acceptor. When the distance separating donor and acceptor is bigger than 2 R_0 , no FRET can occur. From this, it is possible to distinguish between 2 proteins being present in the same cytoplasmic compartment for instance, from those undergoing specific protein-protein interactions.

Sensitized emission (SE) is one of the most used methods for evaluation of FRET efficiencies. It is used on live cells as well as on fixed samples, as long as control samples are available. Because this method is non-invasive, it is most frequently used on live cells experiments, allowing FRET measurements on moving targets.

The method involves measuring the donor and the FRET signal (donor excitation only) in sequence with the detection of the acceptor (acceptor excitation only).

We will describe the technique as steps taken while using the FRET SE approach. This wills involve:

- The measurement of a FRET sample and references of Donor only and Acceptor only samples. Ideally all these conditions should be in the same sample preparation.
- The Donor and Acceptor measurement will give the correction constants for elimination of the excitation and emission cross talk.
- The acquisition of the FRET signal is done in sequential mode (best in line by line).
  Sequence 1: Excitation of the Donor followed by the detection of the Donor and Acceptor (= FRET sample).
  Sequence 2: Excitation of the Acceptor followed by the detection of the Acceptor only.
- The measuring parameters (gain, detection window, excitation wavelength, etc) ***must*** be kept constant.

As described above, this method will need references (donor only and Acceptor only) in order to obtain calibration coefficients to correct for excitation and emission cross talk.

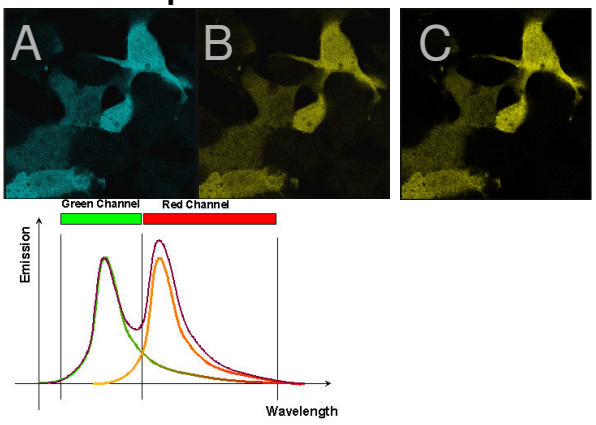
For example, one of the calculation method (method 1 in our Leica FRET SE Wizard) used to calculate these correction factors is based on the formula described by Wouters *et al*, where:

$$E_A(i) = \frac{B - A \times b - C \times c}{C}$$

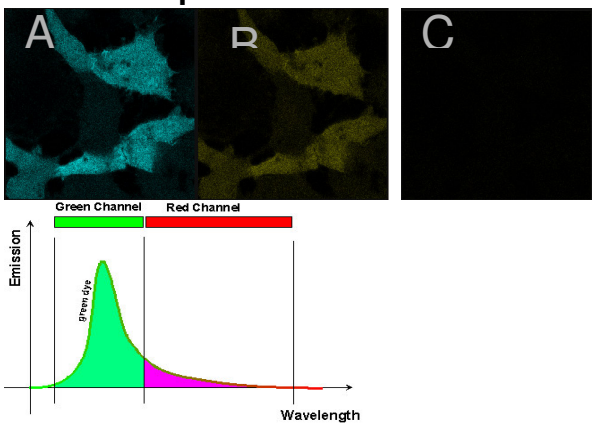
Donor cross-talk correction: A x b; were $b=B^D/A^D$
Acceptor cross-excitation correction: C x c; where $c=B^A/C^A$

Example:

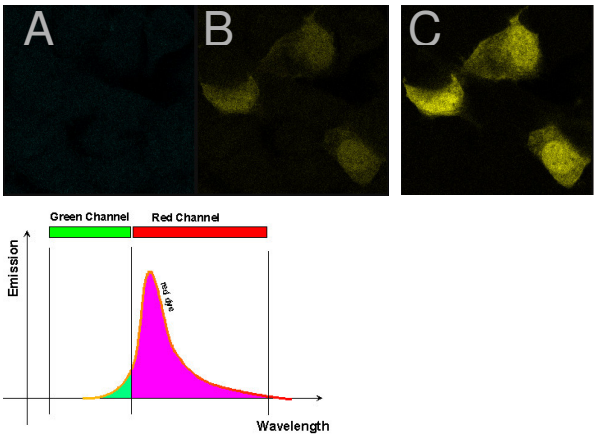
FRET sample



Donor sample



Acceptor sample



Formula for FRET SE

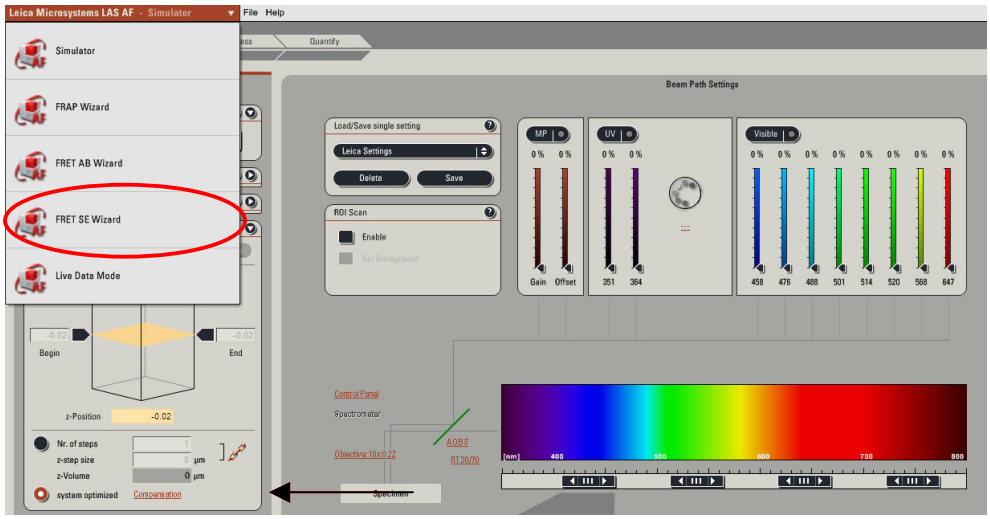
$$E_A(i) = \frac{B - A \times b - C \times c}{C}$$

FRET (B)
Donor emission (A)
Donor emission + crosstalk ratio (b)
Acceptor emission (C)
Acceptor excitation crosstalk ratio (c)

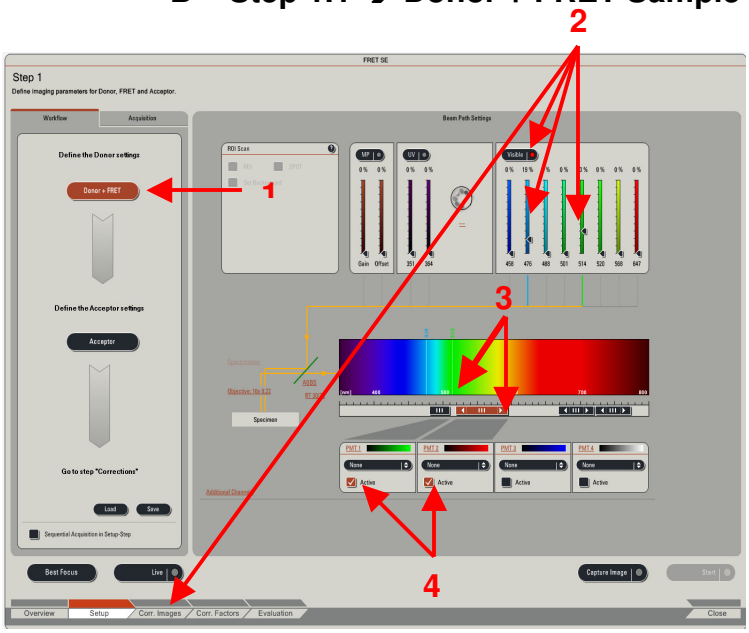
Cos7 cells labeled with CFP and YFP
Courtesy: L. Gelman, CIG, Université de Lausanne

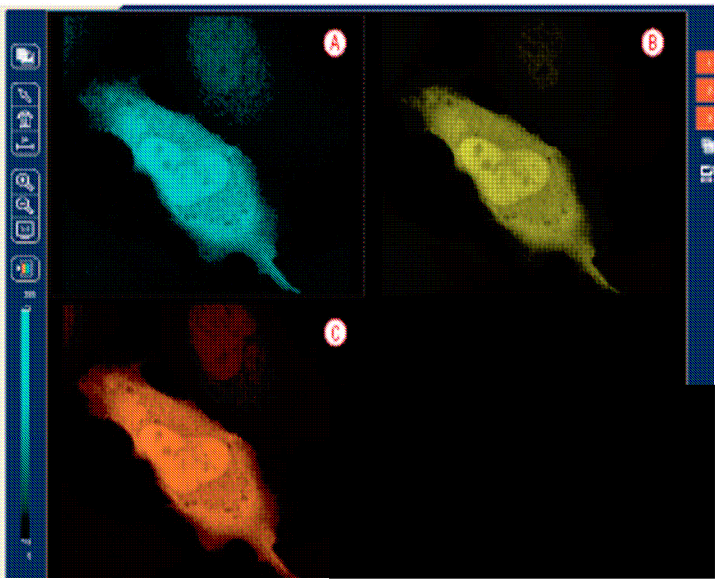
2- FRET SE using LASAF wizard:

A – Activate the FRET SE wizard: Click on the upper left corner on the **Leica Microsystems LASAF** drop-down window to access the applications. Choose FRET SE Wizard as shown on the picture.



B – Step 1.1 ➔ Donor + FRET Sample Settings: (using the FRET sample



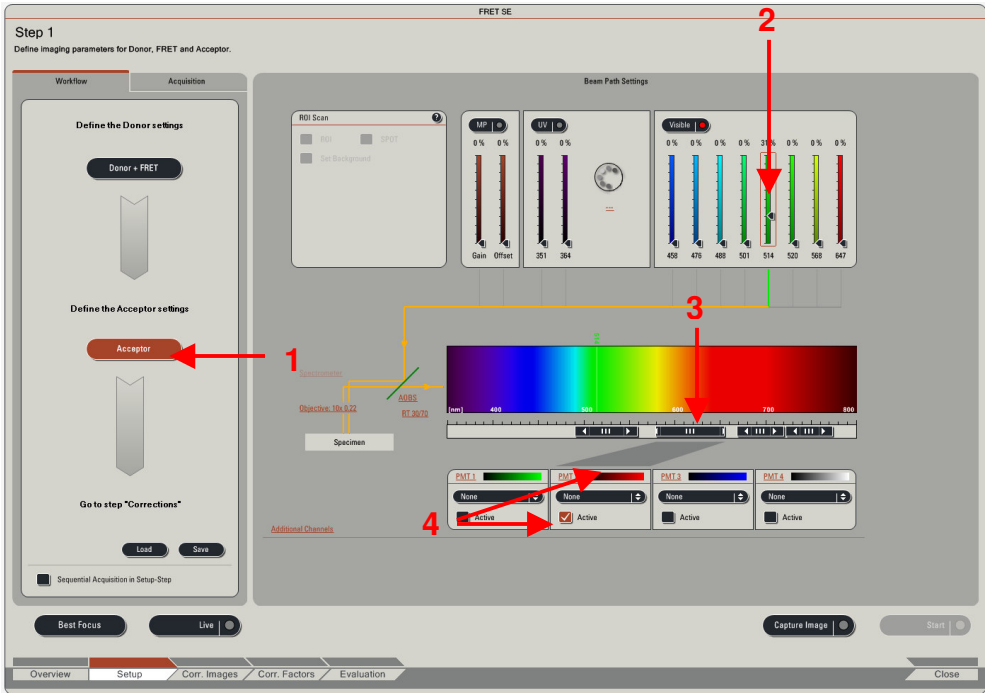


1- Activate the Donor + FRET.
2- Activate the laser line corresponding to your Donor excitation (476 nm laser line in this example = CFP), and the laser line for the Acceptor (514 nm laser line = YFP). This will allows a simultaneous excitation and detection of the donor and acceptor. This will also enable to assess the fluorescence intensities, PMT gain, and laser dose for each fluorochrome. Make all the adjustments by using the **Live** button. Check for appropriate image resolution. You may change the zoom factor via the control panel or via the tools under **Acquisition**.
3- Position your PMTs in accordance with your Donor and Acceptor emissions.
4- Activate your PMTs and change the color if needed by clicking on the color line.

➔ Reduce now the laser light of the Acceptor down to 0%. Re-adjust the acceptor PMT to be slightly below saturation. You are now exciting only selectively the donor and have properly defined your conditions for the donor and FRET imaging.

The images you can see in the viewer are generated using a line by line sequential scan. The first sequence **(A)** + **(B)** consist of the donor signal **(A)** and FRET signal **(B)**. The excitation wavelength is selectively chosen for the donor excitation. The second sequence **(C)** contains the Acceptor signal (C) where the excitation light is selective for the acceptor excitation.

C – Step 1.2 ➔ Acceptor Setup:



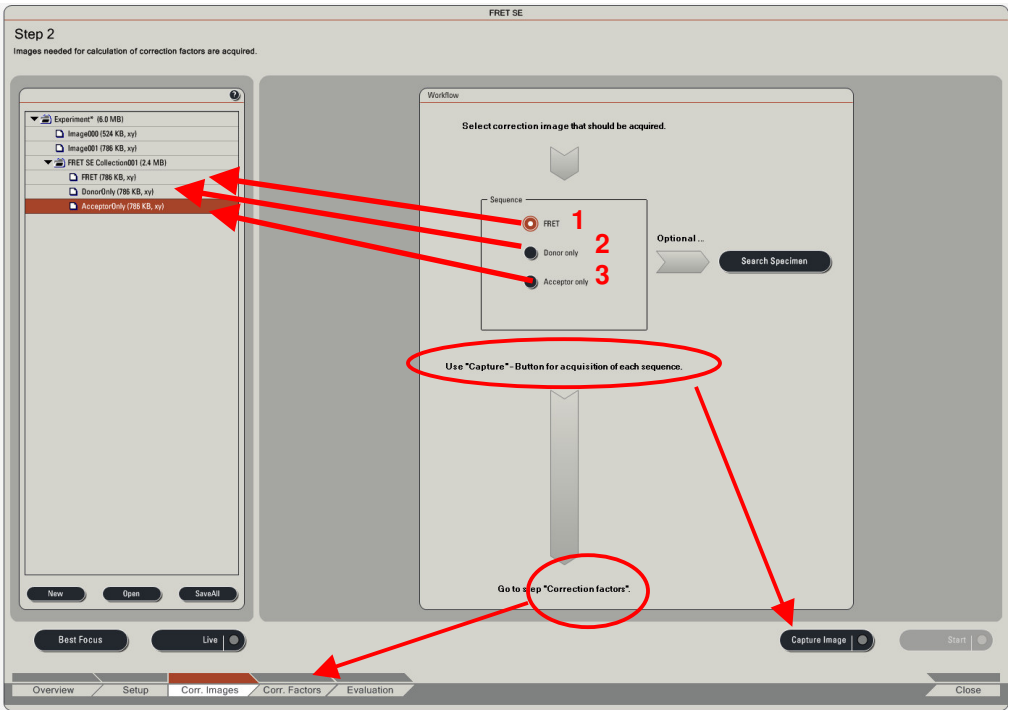
1- Activate the Acceptor.
2- Activate the laser line corresponding to your Acceptor excitation (514 nm laser in this example).
3- Position your PMT in accordance with your Acceptor emission.
4- Activate your PMT and change the color if needed by clicking on the color line.

➔ Slowly increase the excitation light for the acceptor until the signal is just below saturation. Do **NOT** change the PMT gain or offset, as this will also change the imaging conditions for the FRET signal detection.

➔ Define the number of averages for best imaging conditions using the tools under **Acquisition**.

➔ Proceed to the next step **Correction images** to acquire control images of the specimen for calibration

D – Step 2 ➔ Correction Images



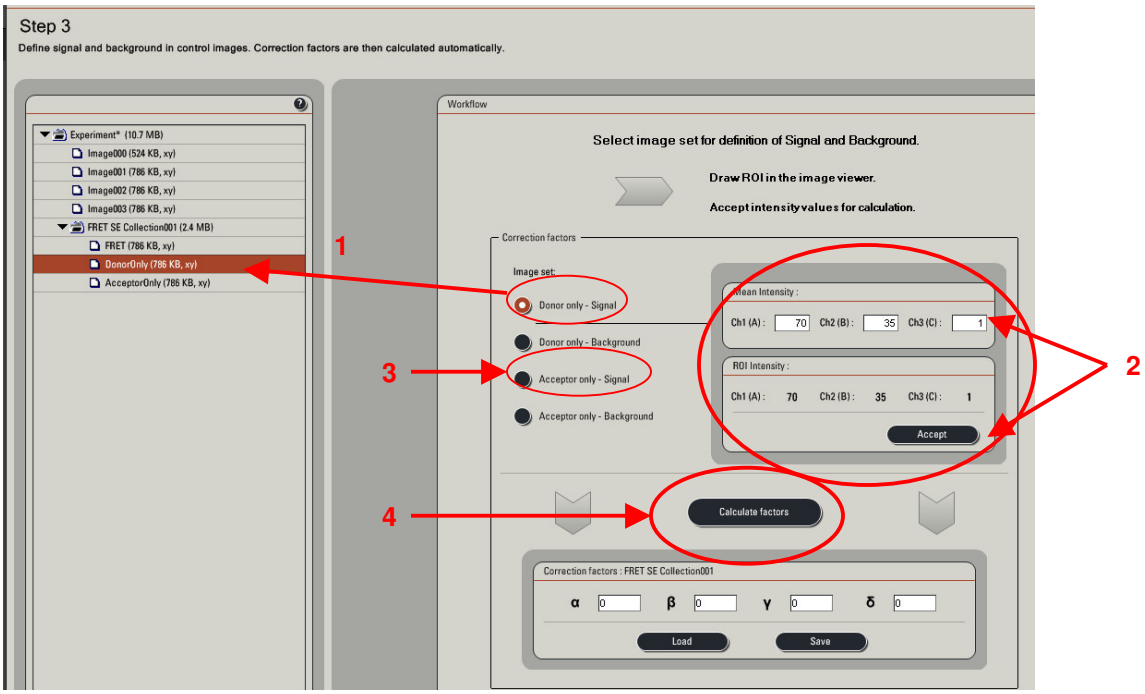
1- Take first an image of your **FRET** sample, since it is already under the scope and correctly setup. Acquire the sequence via **Capture Image**. The image will automatically named **FRET**.
2- Click on the **Donor Only**. Place your **Donor Only** sample under the microscope if your sample doesn't contain a Donor only in the same preparation. Acquire the sequence via **Capture Image**. For a better field of vision you may want to go back to zoom 1 to be able to find adequate cells to properly match the intensities and to avoid saturated regions. The image will automatically be named **Donor Only**.

➔ Click on **Search Specimen** to have access to all the **Acquisition** tools needed. When you exit **Search Specimen** (by clicking on the button "Search Specimen" again), you *will automatically return to the zoom factor and resolution conditions of all previous measurements.*

3- Click on the **Acceptor Only**. Place your **Acceptor Only** sample under the microscope if your sample doesn't contain an Acceptor only on the same slide. Acquire the sequence via **Capture Image**. The image will automatically named **Acceptor Only**. The **Search Specimen** tool is accessible at this step too.

➔ Proceed to the next step **Correction Factors** to generate the calibration factors.

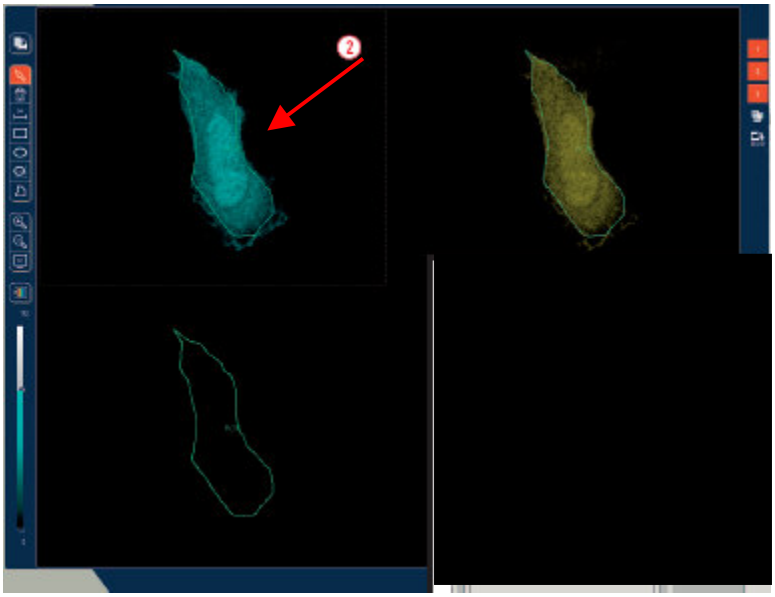
D – Step 3.1 ➔ Correction Factors



1- First, be sure that you have the appropriate image set in the experiment tree to match the image name set in the Correction factor.

2- Draw a region of interest (**ROI**) in the image where only the **Donor** signal is found. The mean intensities within the ROI are shown in the interface. Press **Accept** to use these values for calculation of calibration coefficients.

➔ The background subtraction is optional. It is only necessary if the image show background above 0. If so, then draw a ROI in the image where the background is only present.



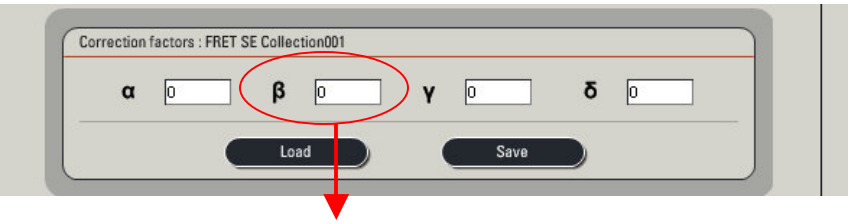
3- Repeat the same process for the **Acceptor Only** sequence.

➔ For a better interpretation of the intensity distribution, the following table can explain what intensity distribution is taken and from which specimen (Donor only, FRET sample, Acceptor only).

	Channel 1 (A)	Channel 2 (B)	Channel 3 (C)
Specimen: FRET	Signal (Donor)	Signal (FRET)	Signal (Acceptor)
Specimen: Donor only	Signal (Donor)	Signal < Channel 1 (x-talk)	No Signal
Specimen: Acceptor only	Very little to no Signal (x-excited x-talk Acceptor)	Signal < Channel 3 (x-Excitation)	Signal (Acceptor)

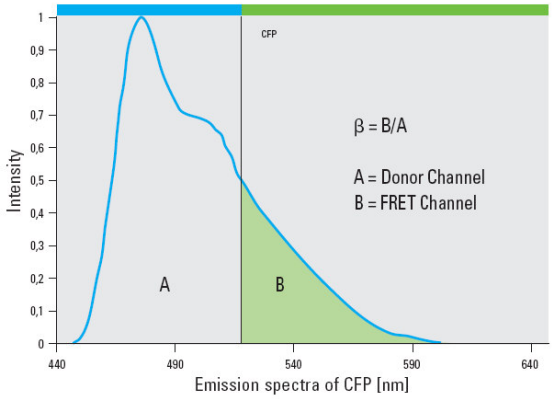
4- Press **Calculate Factors** to generate the correction factors.

D– Step 3.2 ➔ Calibration Calculation and Explanations



Parameter β (or b) is obtained with **donor** only specimen

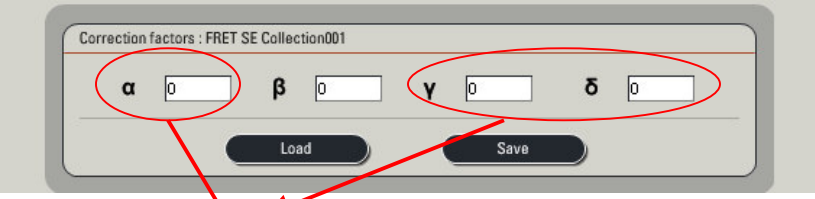
β corrects for donor cross-talk: β = B/A



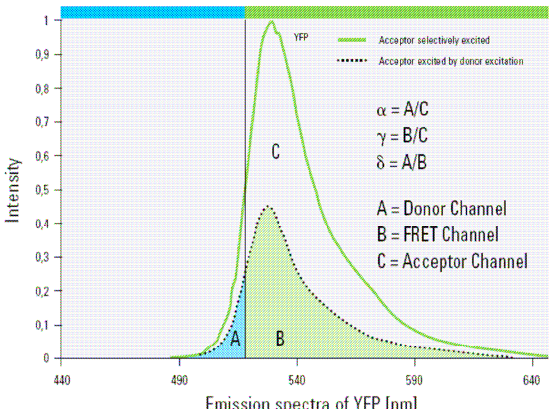
$\beta = B^D/A^D$

A = $Signal_{Donor}$

B = $Signal_{IndirectAcceptor}$



Parameters **α, γ, δ** are obtained with **acceptor** only specimen



$\alpha = A^A/C^A$
$\gamma = B^A/C^A$
$\delta = A^A/B^A$

A = $Signal_{Donor}$
B = $Signal_{IndirectAcceptor}$
C = $Signal_{DirectAcceptor}$

F – Step 4 ➔ Calculations of FRET Efficiency and Live Cell measurements

1- The calculation method can be chosen at this step to calculate the apparent FRET efficiencies. The method 1 is automatically applied (**1**). In any case, the *calculation method can be change after running the experiment*.

Calculation of FRET efficiency:

EA is the apparent FRET efficiency. A, B, C correspond to the intensities of the 3 signals (donor, FRET, acceptor) and α, β, γ and δ are the calibration factors generated by acceptor only and donor only references.

Method 1:

$$E_A(i) = \frac{B - A \times \beta - C \times \gamma}{C}$$

Ref. Wouters et al., TRENDS in Cell Biology, Vol 11, No.5, May 2001: 203-211

Method 2:

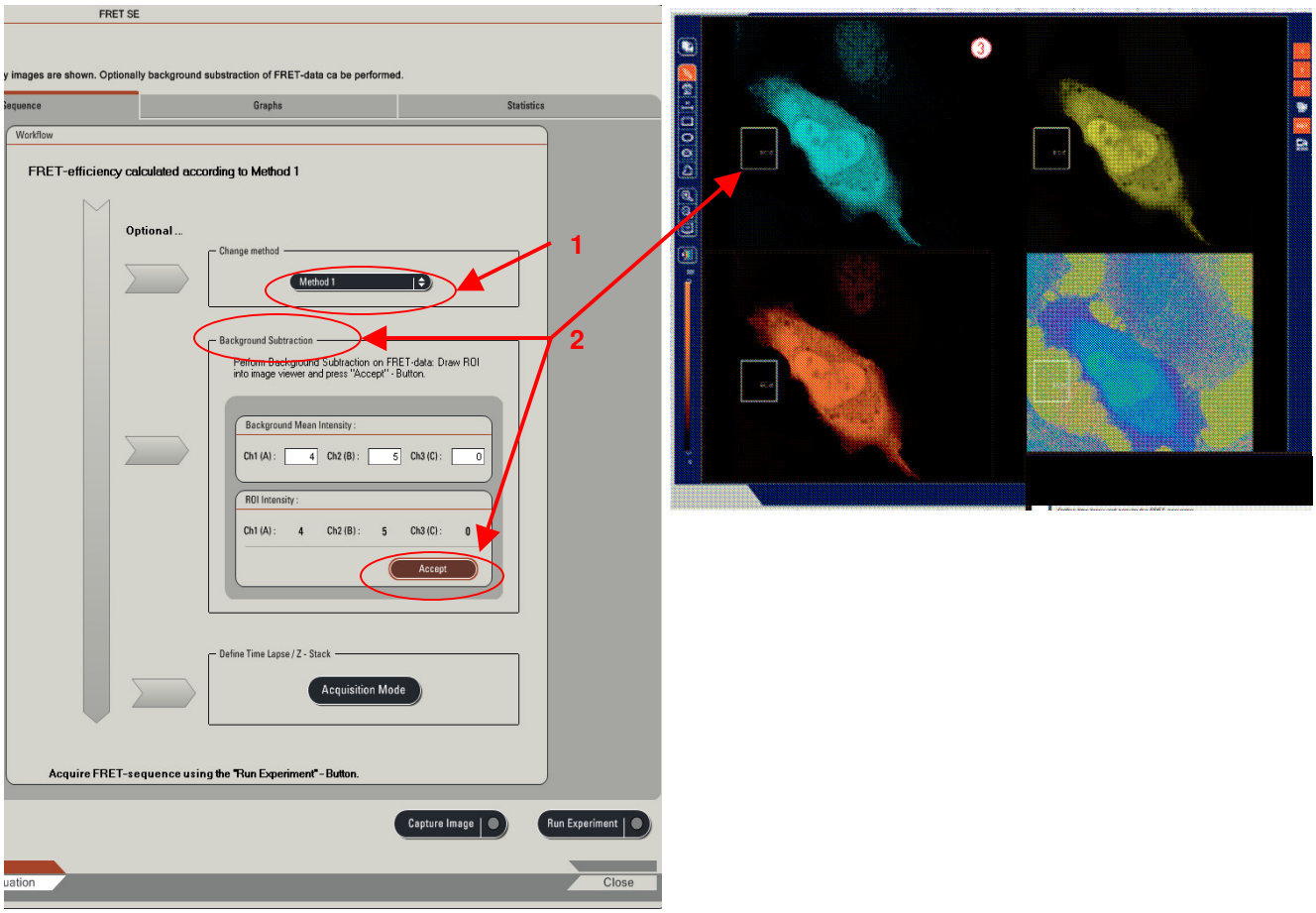
$$E_A(i) = \frac{B - A \times \beta - C \times (\gamma - \alpha \times \beta)}{C \times (1 - \beta \times \delta)}$$

Ref. Van Rheenen, J., M. Langeslag, K. Jalink: Correcting Confocal Acquisition to Optimize Imaging of Fluorescence Resonance Energy Transfer by Sensitized Emission. Biophysical Journal, Vol. 86, April 2004: 1-13.

Method 3:

$$E_A(i) = \frac{B}{A}$$

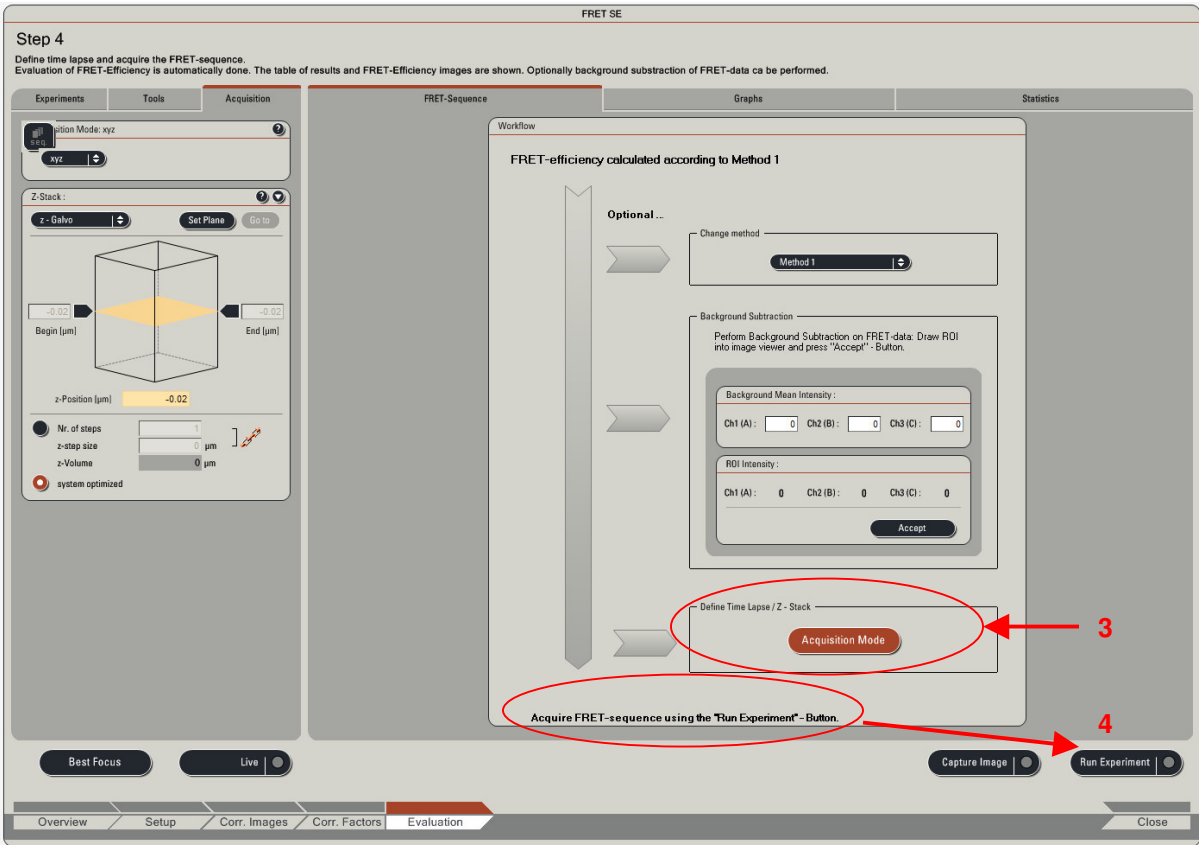
The Ratiometric Calculation is used in samples with a fixed stochiometry (1:1) of donor and acceptor (e.g. Cameleons).



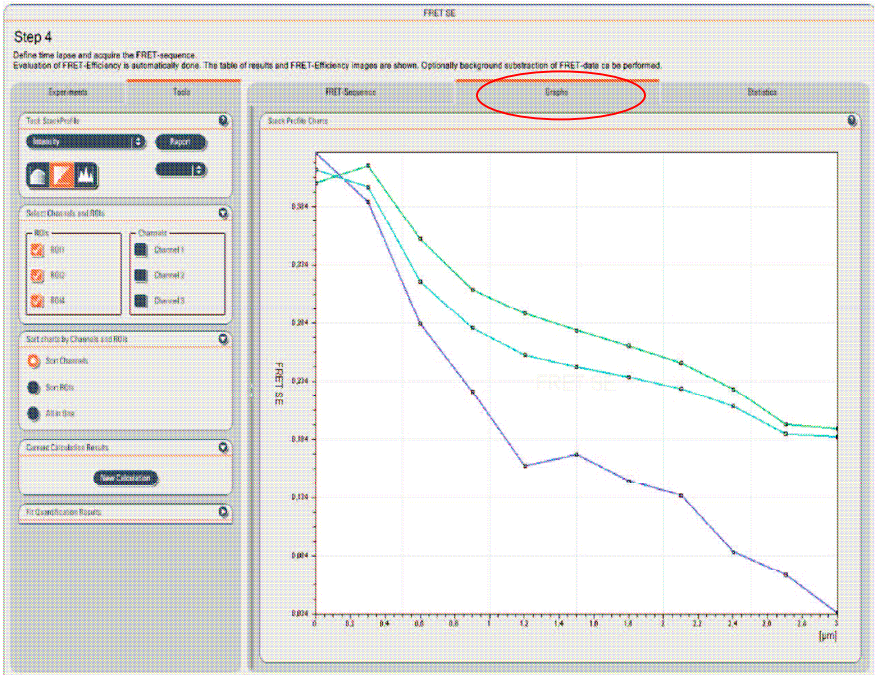
2- The background can be subtracted if above 0 by drawing an ROI (**2**) in the image viewer where only the background is present. Press **Accept** to include the background subtraction into the calculations.

3- A time-lapse experiment can be setup at this moment by clicking on **Acquisition Mode** button (**3**). The Acquisition drop-down window will appear allowing the setup of z-stacks and/or time-lapse series.

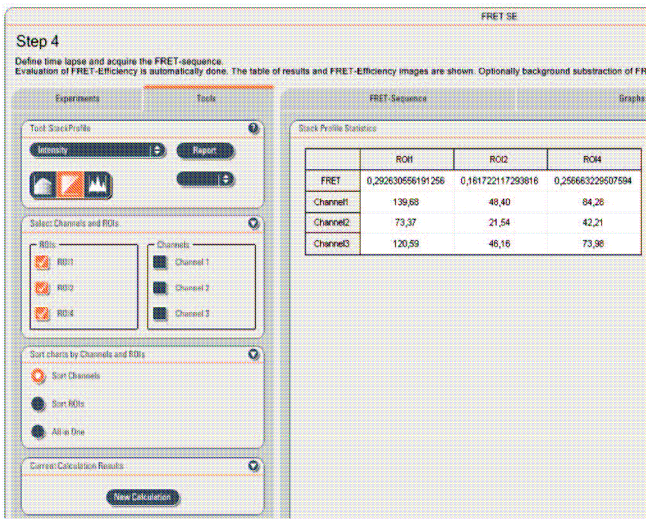
4- Start your series with **Run Experiment** (**4**).

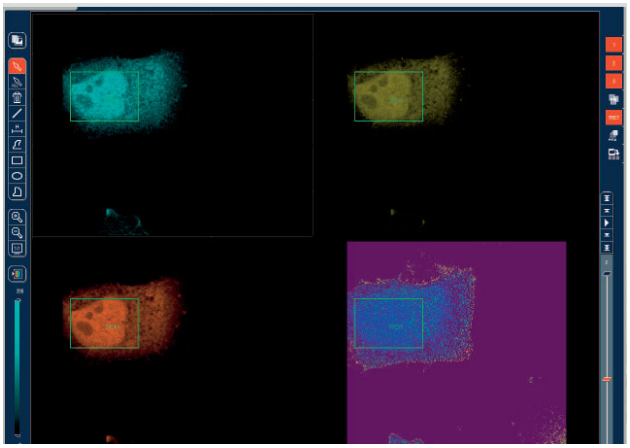


The results of the FRET SE live cell measurements and intensities values are displayed in a graph during the experiments and can be reviewed while recording the series by clicking on the **Graph** Tab. The FRET efficiencies values are displayed in the **Statistics** tab sheet.



FRET SE efficiencies carried out on fixed specimen can be analyzed under **Statistics**. No graphical display will be available.
Under the **Statistics** tab, ROIs can be drawn to choose the appropriate regions of interest in the image.





References:

Fred S. Wouters, Peter J. Verveer and Philippe I. H. Bastiaens, Imaging biochemistry inside cells, Trend Cell Bio. 2001 May; 11(5): 203-11.

Van Rheenen J., Langeslag M., Jalink K. Correcting confocal acquisition to optimize imaging of fluorescence resonance energy transfer by sensitized emission. Biophy. J. 2004 Apr; 86(4): 2517-29.